

REMARKS

At this time, applicants thank Examiner Mertz for her time and consideration in discussing the present application with the undersigned on several occasions.

Claims 89-91 and 113-116 are pending in the present application. Support for the changes to claims 89-91 are supported generally throughout the specification. In particular, support may be found in the present specification at pg. 48, third full paragraph. Claims 89-91 have also been amended to address an informality found within the claims. Support for amended claims 113-116 may be found in the present specification at pg. 49, third full paragraph. Claims 1-88, 92-112, and 117-118 have been canceled.

In the outstanding Official Action, claims 89-91 and 101-106 were rejected under 35 USC 112, first paragraph, for allegedly not satisfying the enablement requirement. This rejection is respectfully traversed.

In a non-narrowing amendment, claims 89-91 recite a method for culturing blood precursor cells and/or hematopoietic stem cells in vitro, comprising contacting said cells in vitro with Stem Cell Factor (SCF) and a recited serrate-1 peptide. Claims 113-116 recite a method for culturing CD 34 positive blood precursor cells in vitro, comprising contacting said cells in vitro with SCF and recited serrate-1 peptide. While applicants believe that the present non-narrowing amendment obviates this

rejection, applicants do not disclaim any potential applications (e.g., suppression of colony formation, proliferation, differentiation, etc...) for the claimed methods.

As to the cells utilized in Example 10, applicants submit that one skilled in the art would understand the scope and meaning of CD34 positive cells are stem cells. Accordingly, applicants submit an article with the present amendment explaining the structure, biology, and clinical utility of CD34 (KRAUSE et al., pgs. 8-9).

Thus, in view of the above, applicants respectfully request that the rejection be withdrawn.

Claims 89-91 and 101-118 were rejected under 35 USC 112, first paragraph, for allegedly not satisfying the enablement requirement. This rejection is respectfully traversed.

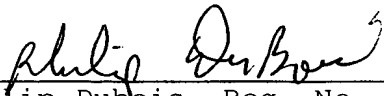
As noted above, claims 89-91 and 113-116 provide that the recited cells are cultured with SCF and the recited peptide. The claims do not exclude and applicants do not disclaim the possible presence of other compounds with SCF and the recited peptide. As a result, applicants respectfully request that the rejection be withdrawn.

In view of the present amendment and foregoing Remarks, therefore, applicants believe that the present application is in condition for allowance at the time of the next Official Action. Allowance and passage to issue on that basis is respectfully requested.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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**APPENDIX**

- Krause et al, *CD34: Structure, Biology, and Clinical Utility*, Blood Vol. 87, No. 1 (1996).

# BLOOD

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## REVIEW ARTICLE

### CD34: Structure, Biology, and Clinical Utility

By Diane S. Krause, Mary Jo Packler, Curt I. Civin, and W. Stratford May

**C**D34 IS A surface glycoprophosphoprotein expressed on developmentally early lymphohematopoietic stem and progenitor cells,<sup>1-3</sup> small-vessel endothelial cells,<sup>4,5</sup> and embryonic fibroblasts.<sup>6</sup> CD34<sup>+</sup> bone marrow (BM) cells comprise only 1.5% of marrow mononuclear cells, but contain precursors for all lymphohematopoietic lineages, as evidenced by the finding that CD34<sup>+</sup> cells purified from marrow can reconstitute hematopoiesis of primates, humans, or mice undergoing autologous marrow reinfusion after myeloablative therapy (BM transplant<sup>7-9,11</sup>). CD34<sup>+</sup> hematopoietic cells obtained from marrow or blood are in clinical use in transplantation and gene therapy studies, including ongoing attempts to expand hematopoietic stem/progenitor cells *in vivo*. Despite the importance of CD34 as a marker of early hematopoietic stem/progenitor cells in experimental and clinical hematopoiesis, the function of CD34 is not yet clear. Because of its potential role in such fundamental processes as hematopoietic stem/progenitor cell development and inflammation, studies on the regulation and function of CD34 are being pursued in several laboratories.

Recent experiments on the function of CD34 indicate that CD34 expressed on endothelial cells may play a role in leukocyte adhesion and "homing" during the inflammatory process, and it has been hypothesized that CD34 plays a role in stem/progenitor cell localization/adhesion in the BM.<sup>10,17</sup> CD34 may also be involved in maintenance of the hematopoietic stem/progenitor phenotype.<sup>18</sup> This review will concentrate on our current knowledge of the expression, structure, regulation, function, and clinical utility of the CD34 molecule.

#### CD34 MONOCLONAL ANTIBODY (MoAb) DISCOVERY AND EXPRESSION OF CD34 ON HEMATOPOIETIC CELLS

CD34 was discovered as the result of a strategy to develop antibodies that specifically recognize small subsets of human marrow cells, but not mature blood or lymphoid cells.<sup>1-4,19,20</sup> The first CD34 antibody, My10, was the product of a hybridoma generated from a mouse immunized with the KQ1a myeloid leukemia cell line.<sup>1,21</sup> CD34 antibodies specifically detect an average of only 1.5% of low-density mononuclear cells from BM aspirates of normal donors.<sup>1,22</sup> In contrast, there is less than 0.5% CD34 labeling of peripheral blood (PB) cells.<sup>23</sup> The CD34<sup>+</sup> cell population from normal human BM is enriched in morphologic blast cells, in contrast to the CD34<sup>+</sup> cell fraction, which contains predominantly recog-

nizable precursor cells at diverse maturational stages of hematopoiesis.

The discovery of CD34 as a hematopoietic cell-surface antigen has transformed and accelerated studies into developmental hematopoiesis. Immunoaffinity-purified CD34<sup>+</sup> marrow and cord blood cells are 10- to 100-fold enriched in colony-forming units (CFUs), whereas CD34<sup>+</sup> cell populations are depleted. The CD34<sup>+</sup> cells include CFU-macrophage (CFU-M), CFU-granulocyte (CFU-G), CFU-GM, burst-forming units-erythroid (BFU-E), and are particularly enriched in the earliest types of colony-forming cells, such as CFU-mix and CFU-blast.<sup>24</sup> A fraction of the most mature unipotent CFU, such as CFU-E and CFU-G, can be found in the CD34<sup>+</sup> cell fraction. There are two distinct populations of CD34<sup>+</sup> BM cells that differ in their relative levels of surface CD34 expression by flow cytometric analysis.<sup>3,11,22,25</sup> The CD34<sup>high</sup> population contains the majority of the immature hematopoietic progenitor cells, whereas the CD34<sup>low</sup> population contains more lineage-committed progenitors, as assayed *in vitro*. The CD34<sup>high</sup> population is enriched for unipotent progenitors, including CFU-GM, BFU-E, CFU-E, CFU-megakaryocyte (CFU-Meg), and even more highly enriched for the developmentally early multipotential CFU-GEMM (CFU-mix), CFU-blast, and long-term culture ini-

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*Becton Dickinson Corporation and Baxter HealthCare Corporation provided research funding for portions of C.I.C.'s research cited in this review article. C.I.C. is the inventor of the CD34 monoclonal antibody and related inventions, and the Johns Hopkins University holds patents on these inventions. Under licensing agreements between the University and these companies, C.I.C. is entitled to a share of the sales royalty received by the University. C.I.C. has also served as a consultant for these companies. The terms of these arrangements have been reviewed and approved by the University in accordance with its conflict of interest policies.*

*D.S.K. is a Leukemia Society of America Fellow.*

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using cells (LT-CIC<sup>7,11,26</sup>). The LT-CIC and CFU-bias<sup>27</sup> assays are thought to be the most physiologically relevant *in vitro* assays for the putative stem cell, although these cells may be functionally distinct from the true stem cell. Recent data indicate that CD34 is expressed on the population of pluripotent progenitors that can be enriched from human BM after selective removal (by 5-fluorouracil treatment *in vitro*) of committed progenitors stimulated to divide by interleukin-3 (IL-3) and stem cell factor.<sup>28</sup> Very immature lymphoid cells are also CD34<sup>+</sup>. Cells that express terminal deoxynucleotidyl transferase, which marks the stage when lymphoid cells rearrange the Ig (B cells) or antigen receptor (T cells) genes, are CD34<sup>+</sup>.<sup>22,23</sup> Early CD19<sup>+</sup>, CD10<sup>+</sup> B cells express CD34, whereas later CD20<sup>+</sup> B cells are CD34<sup>-</sup>. Taken together, this suggests that CD34 is expressed at high levels on the earliest hematopoietic cells and surface expression decreases to undetectable levels by the stage when maturing hematopoietic cells lose the capacity to form colonies *in vitro*.<sup>24</sup> The stage-specific expression of CD34 in both human and murine hematopoietic stem/progenitor cells suggests a potential regulatory role for CD34 in the early phases of developmental hematopoiesis.

The expression of murine CD34 seems to parallel that of human CD34 on hematopoietic cells. As with human CD34, murine CD34 is expressed at both low and high levels on BM cells.<sup>11</sup> CD34<sup>int</sup> murine BM cells are enriched in hematopoietic progenitors as assayed by CFU-GEMM, CFU-GM, and BFU-E. Just 200 CD34<sup>+</sup> BM cells are capable of long-term reconstitution of hematopoiesis in lethally irradiated recipients, indicating the presence of hematopoietic stem cells.<sup>11</sup> Additional studies using other antibodies against murine CD34 should be performed to firmly establish that murine CD34 is truly similar to human CD34 on hematopoietic cells.

As stated above, BM transplantation (BMT) studies in primates, including mice,<sup>11</sup> baboons,<sup>9</sup> rhesus monkeys,<sup>10</sup> and humans,<sup>9</sup> indicate that a CD34<sup>+</sup> subpopulation of total BM cells can provide durable donor-derived long-term host lymphohematopoietic reconstitution. Thus, CD34 enables the identification and purification of hematopoietic progenitor cells, by means of a single reagent. Definitive proof that CD34 is expressed on human hematopoietic stem cells awaits the long-term outcomes of clinical BMT trials using CD34<sup>+</sup> cell allografts (see below). If the allogeneic donor CD34<sup>+</sup> population (as opposed to host stem cells) provides durable, long-term multilineage lymphohematopoiesis in humans, this will imply that these cells are capable both of self-renewal and multilineage differentiation, and thus satisfy the most stringent definition of the stem cell.

#### EXPRESSION OF CD34 BY NONHEMATOPOIETIC CELLS

Various tissues and cell lines have been studied for CD34 expression. Detection of steady-state CD34 expression has been performed either by Northern analysis of total RNA or Southern analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products. In some cases, Western blotting or immunohistochemistry has been performed to detect protein expression. A compilation of the published results is shown in Table 1. In addition to its expression on murine

Table 1. CD34 Expression Profile

| Cell Lines               | Protein Detection*          | mRNA Detection* | References           |
|--------------------------|-----------------------------|-----------------|----------------------|
| Human: KG1, KG1a         | +                           | +               | 1, 7, 42, 80         |
| KMT-2                    | +                           | +               | 16, 42               |
| KPM-8402                 | +                           | +               | 42, 67               |
| MOLT-13                  | +                           | +               | 51, 57               |
| AML-1                    | +                           | +               | 7, 42                |
| EA hy828                 | ND                          | +               | 42                   |
| UT7                      | ND                          | +               | 51                   |
| Bovine: BAE              | ND                          | +               | 42                   |
| Murine: ES cells         | +                           | +               | 11, 131              |
| NIH 3T3                  | +                           | +               | 8, 11                |
| Swiss 3T3                | ND                          | +               | 8                    |
| PA-8                     | +                           | +               | 11, 52               |
| DA-1                     | - (10)                      | +/-             | 11, 52               |
| IC 2                     | ND                          | +               | 52                   |
| M1                       | +                           | +               | 11, 52               |
| Primary malignant tissue |                             |                 |                      |
| Human: AMLs (~40%)       | +                           | +               | 7, 51                |
| B-ALL (~70%)             | +                           | ND              | 7                    |
| T-ALL (~5-20%)           | +                           | ND              | 7, 29                |
| MDS                      | +                           | ND              | 51, 57               |
| CMIL-CP                  | +                           | ND              | 51                   |
| Angiosarcoma             | +                           | ND              | 16                   |
| Kaposi's sarcoma         | +                           | ND              | 31, 32               |
| Hepatic hemangioma       | +                           | ND              | 16                   |
| Primary normal tissue    |                             |                 |                      |
| Human: Bone marrow       | +                           | +               | 1, 8, 9, 23, 28, 132 |
| Cord blood               | +                           | ND              | 133-138              |
| Peripheral blood         | +                           | ND              | 109-112              |
| Endothelial cells        | +                           | ND              | 4, 6, 16, 20, 139    |
| Fetal bone marrow        | +                           | ND              | 132, 140, 141        |
| Heart†                   | - (vasc)                    | +               | 7, 51                |
| Skeletal muscle†         | - (vasc)                    | +               | 7, 51                |
| Kidney†                  | - (vasc)                    | +               | 7, 51                |
| Placenta†                | - (vasc)                    | +               | 6, 51                |
| Lung†                    | - (vasc)                    | +               | 7, 51                |
| Brain†                   | +                           | +               | 7                    |
|                          | (vasc and rare glial cells) |                 |                      |
| Murine: Bone marrow      | +                           | +               | 8, 11, 52            |
| Endothelial cells        | +                           | ND              | 13                   |
| Fetal liver              | ND                          | +               | 52                   |
| Liver†                   | - (vasc)                    | +               | 6, 13, 52            |
| Spleen†                  | - (vasc)                    | +               | 6, 13, 52            |
| Thymus†                  | - (vasc)                    | +               | 6, 13, 52            |
| Testis†                  | ND                          | +               | 6, 52                |
| Kidney†                  | - (vasc)                    | +               | 13                   |
| Brain†                   | +                           | +               | 6, 13, 30, 52        |
|                          | (vasc and rare glial cells) |                 |                      |

Cell lines negative for CD34 include KB2, NH00, CEM, Daudi, U937, HeLa, P815, ST2, WEHI-231, FDCP2, NFS-60.

Abbreviation: (vasc), immunohistochemistry studies in which CD34 expression was exclusively on vascular endothelial cells.

\* Protein detected either by immunohistochemistry or FACS. mRNA detected by either Northern analysis or RT-PCR.

† Only RT-PCR has been used.

‡ RNA extracted from whole tissue which includes parenchyma and aspirary endothelium.

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and human hematopoietic stem/progenitor cells, CD34 is expressed by virtually all small-vessel endothelial cells.<sup>4,5</sup> Expression of CD34 mRNA has also been detected in liver, spleen, BM, and thymus,<sup>6</sup> as well as in both fetal and adult heart, lung, skeletal muscle, kidney, and brain.<sup>4</sup> However, because endothelial cells contained in these tissue samples may be a contaminating source of CD34 mRNA, CD34 expression in the parenchymal cells of these tissues awaits confirmation by a more precise method, such as immunohistochemistry. Using a polyclonal antimurine CD34 antiserum, the distribution of CD34 in adult mouse tissues has recently been studied.<sup>12</sup> In addition to demonstrating strong endothelial staining, staining of some nonendothelial cells in the brain and kidney was observed. Although these findings suggest that CD34 expression may be more widespread than initially thought, specificity for CD34 was not shown. Staining of neural tissue has also been observed using a newly developed MoAb against murine CD34 that binds nonglycosylated intracellular CD34.<sup>20</sup> Most solid tumors are CD34<sup>+</sup>, except for malignancies of vascular origin such as Kaposi's sarcoma.<sup>4,7,31-33</sup>

## CHARACTERIZATION OF CD34 ANTIBODIES

Anti-CD34 MoAbs were first clustered in the Third International Differentiation Workshop, then more extensively categorized in the CD34 cluster workshop of the Fourth and Fifth International Differentiation Workshops.<sup>7,14,34,35</sup> Seven MoAbs were studied in the Fourth Workshop<sup>7</sup>; My10,<sup>1</sup> BL3C5,<sup>2</sup> 12.8 and 115.2,<sup>3</sup> ICH3,<sup>20</sup> and TUK3<sup>19</sup> were raised against the acute myelogenous leukemia-derived KG1 or KG1a cell lines,<sup>20</sup> while QBEND10 was raised against human placental endothelial cells.<sup>4</sup> The binding epitopes of each MoAb on the heavily glycosylated CD34 protein were found to be differentially sensitive to neuraminidase (NA), which cleaves sialic acid residues, and chymopapain (CP) or *Pasteurella hemolytica*-derived glycoprotease (GP). GP selectively cleaves sialylated O-linked glycans. Based on this, the MoAbs have been divided into three classes. Class I antibodies (eg, My10, BL3C5, 12.8, ICH3) are directed against epitopes which are sensitive to NA, CP and GP. Class II antibodies are represented by QBEND10, which detects an epitope sensitive to CP and GP but not NA. Class III antibodies include TUK3 and 115.2, whose epitopes are insensitive to these enzymes. Therefore, each class has a unique epitope within the extracellular domain of CD34. In one method of CD34<sup>+</sup> cell purification, after class I antibody is used to immunopurify the CD34<sup>+</sup> cells, enzymatic cleavage is used to detach the cells from the immunoadfinity substrate.<sup>17,20</sup> Epitope mapping has been confirmed and extended in the Fifth Workshop.<sup>35</sup>

Rabbit and murine CD34 antisera have recently been raised.<sup>11,13</sup> One such antiserum designated Ab 1202 is directed against a recombinant fusion protein containing the extracellular domain of murine CD34.<sup>11</sup> This antiserum has been used to characterize the stem/progenitor cell specificity of CD34 present on murine BM cells. Another antiserum designated Ab 1241 is directed against a C-terminal peptide contained in the intracellular region of murine CD34 (ENC-TQQTSTRNGHSAR<sup>11</sup>). An antibody (Ab902) to a similar

peptide contained in human CD34 has also been raised.<sup>39</sup> Because the C-terminal peptide is present in the full-length but not the truncated form of CD34, this antiserum has been useful for specific detection of the full-length form of the protein<sup>11,39</sup> (Fig 1). A third antiserum raised against murine CD34 was obtained by immunizing rabbits with a recombinant fusion protein containing the extracellular domain of CD34 and the Fc portion of IgG.<sup>12</sup> This antiserum has been used to characterize the distribution of the CD34 protein expressed on murine endothelial cells and nonvascular tissues.<sup>12</sup> Finally, an MoAb designated RAM34 was recently produced, and is commercially available.<sup>40</sup> However, biochemical characterization studies have not yet been reported for RAM34.

## CHARACTERIZATION OF CD34 GENES AND CD34 PROTEIN STRUCTURE

## Cloning CD34 Genes

Both human and murine CD34 cDNAs, as well as the corresponding genomic DNAs, have been cloned.<sup>44-46</sup> Recently, canine CD34 cDNA was also cloned.<sup>45</sup> The human and murine genes consist of nine exons (exons 1 through 8 and exon X) that are contained in a 22- (murine) or 27-kb (human) region of genomic DNA (Fig 1). Overall the genes are highly homologous and evolutionarily well-conserved. Homology is highest in the intracellular domain (90% amino acid identity) encoded by exon 8, and lowest in the N-terminal region of the extracellular domain (43% amino acid identity). The characteristic features of human and murine CD34 are presented in Table 2. The human gene has been mapped to chromosome 1q32 (Table 1).<sup>44,47</sup> Murine CD34 is also on the long arm of chromosome 1.<sup>44</sup> Interestingly, the human 1q32 region contains several genes encoding adhesion matrix and complement cascade-binding molecules, such as LAM-1/CMP140, laminin  $\beta 2$ , and the RNA gene cluster.<sup>48,49</sup> The genetic colocalization of CD34 with adhesion molecules suggests potential coordinate regulation of expression, and therefore may have functional relevance. Despite the fact that CD34 is expressed in a high proportion of acute leukemias, neither rearrangement of the CD34 locus nor point mutations have been reported in any malignancies.

## CD34 Splice Variants

There are two species of CD34 mRNA, derived from an alternative splicing mechanism.<sup>41,50</sup> One species contains exons 1 through 8, and encodes the full-length protein with an intracellular region comprised of 73 amino acids (Fig 1A and B). The resulting full-length human and murine proteins are predicted to contain 385<sup>40,51</sup> and 382<sup>42,52</sup> amino acids, respectively. The splice variant results from the insertion of an additional exon (ie, exon X, which is 194 bp in the human or 156 bp in the murine genes) between exons 7 and 8. Exon X introduces a stop codon and results in the translation of a protein with a shorter cytoplasmic domain comprised of just 16 amino acids. Therefore, while the intracellular cytoplasmic domains differ, the extracellular regions of the two forms of CD34 are predicted to be identical. Thus, any differential function between these two forms of CD34 would likely result from differences in their respective cytoplasmic

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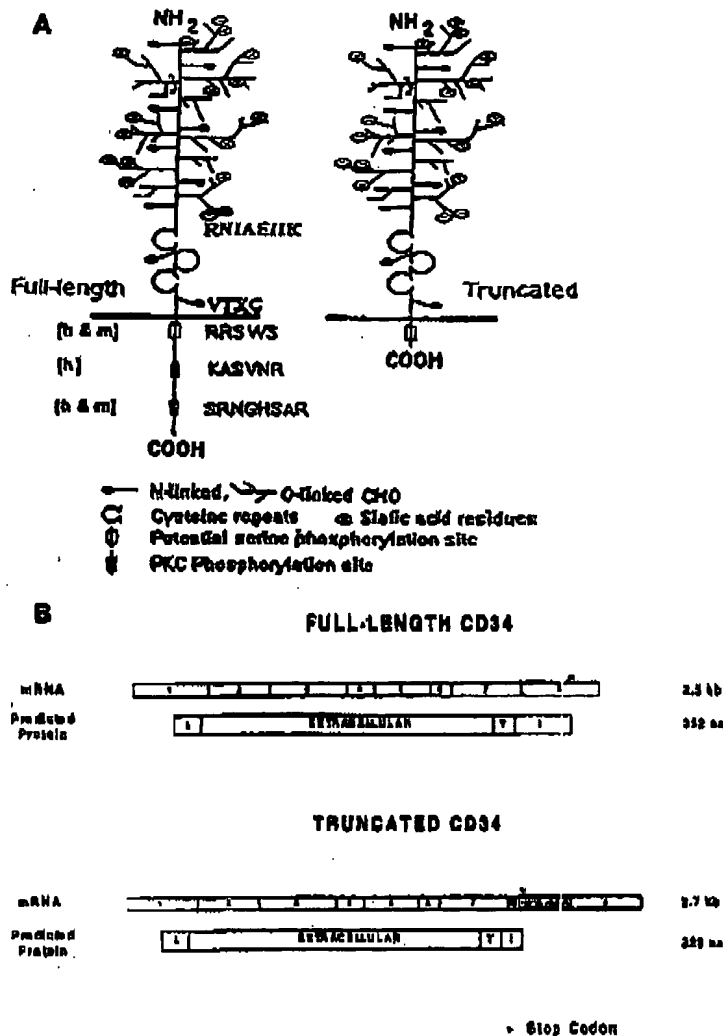


Fig 1. CD34 model and structural properties: (A) Schematic depicting full-length and truncated forms of CD34 including predicted glycosylation patterns and serine phosphorylation sites in human (h) and murine (m) CD34. The truncated form of CD34 is shown for comparison. RNIAEIKK and VTXG are conserved minimal putative recognition sites that may confer adhesion properties (see text for details). (B) Schematic depicting mRNA splice modification mechanisms resulting in translation of a full-length or truncated CD34 protein.

tail regions. For example, the truncated protein lacks potential target sites for protein kinase C (PKC) that are present in the full-length CD34 protein. Both forms of the CD34 protein have been identified in murine and human cells.<sup>11,30</sup> Both the full-length and truncated human CD34 proteins appear to have a mobility of approximately 116 kD on denatured polyacrylamide gel electrophoresis.<sup>30</sup> Although the relative mobility of murine CD34 varies slightly depending on the cell type in which it is expressed, the full-length form is approximately 110 kD, and the truncated form has a mobility of approximately 100 kD.<sup>11</sup>

Normal human<sup>31</sup> and murine (M.J.F., W.S.M., unpublished findings, October 1993) BM cells contain both forms of the CD34 transcript, as determined by RT-PCR analysis. Although the significance of the two CD34 protein forms is not yet clear, preliminary evidence suggests that expression

may vary according to the stage of differentiation of CD34<sup>+</sup> hematopoietic cells (M.J.F., W.S.M., unpublished observations, October 1993). If the cytoplasmic domain plays a role in cell signaling, then a change in the ratio of the full-length to the truncated protein and/or the exclusive expression of one or the other form might modulate this effect of CD34. Interestingly, in purified CD34<sup>+</sup> leukemic blast cells isolated from three patients with acute myelogenous leukemia (AML), only the mRNA species encoding full-length CD34 was expressed.<sup>31</sup> The significance and generality of this finding is not yet clear.

#### Predicted Structural Features of the CD34 Protein

CD34 is a heavily glycosylated type I transmembrane protein that is a member of the sialomucin family of surface molecules (see Table 2 and Fig 1). Based on the predicted



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Table 2. CD34 Characteristics

|                                    | Human                  | Murine                        |
|------------------------------------|------------------------|-------------------------------|
| HPC/tumor cells                    | +                      | +                             |
| Vascular endothelial cells         | +                      | +                             |
| Brain and testis                   | +                      | +                             |
| mRNA length                        | 2.5-2.7 kb             | 2.5-2.7 kb                    |
| mRNA splice variants (73 v 10 sp)  | +                      | +                             |
| Protein products splice variants   | +                      | +                             |
| Genomic organization               | 8 exons + x (7.6) exon | 8 exons + x (7.6) exon        |
| Chromosomal location               | 1q32                   | 1                             |
| Predicted 1 <sup>st</sup> sequence | 382 aa, 39 kD          | 384 aa, 39 kD                 |
| Immunopt. ab.                      | +                      | +                             |
| M, SDS-PAGE                        | 105-120 kD             | 80-kD form<br>105-116-kD form |
| M, neuraminidase treated           | 160 kD                 | 125-130 kD                    |
| Predicted phosphorylation sites    | PKC, GSK, CKII, Cal K  | PKC, GSK, CKII, Cal K         |
| Glycosylated (N-, O-linked sugars) | +                      | + (7 N-, O-linked)            |

Abbreviations: aa, amino acids; Immunopt. ab., immunoprecipitation antibodies available.

amino acid sequence, the CD34 protein shows no strong homology to other proteins currently entered into the database. No enzymatic domain motif is found in the CD34 amino acid sequence, apparently eliminating a role as a protein kinase or phosphatase. However, several small domains within CD34 have homology to known proteins.

Beginning at the NH<sub>2</sub>-terminus, the heavily glycosylated extracellular region of CD34 is similar to that of leukosialin (CD43), a hematopoietic cell-specific sialoglycoprotein expressed by both human<sup>63</sup> and murine<sup>64</sup> leukocytes. CD43 appears to play a role in both adhesion and cellular activation, but its precise function is not yet known. Interestingly, both CD34 and CD43 are substrates for phosphorylation by PKC,<sup>16,22,23</sup> suggesting a possible role for both molecules in transmembrane signaling. Other structural features shared by CD34 and known proteins include similarities between the N-terminal glycosylated regions of CD34 and CD45, the leukocyte common antigen and a protein tyrosine phosphatase.<sup>59</sup> CD34 also displays weak similarities to cell adhesion molecules, including LAM-1,<sup>65</sup> ELAM-1,<sup>66</sup> and membrane cofactor proteins (MCP).<sup>61</sup> The extracellular sequence of CD34 contains two minimal putative adhesion recognition sequences<sup>62</sup> (Fig. 1). The first corresponds to the VTXG sequences contained in thrombospondin and circumsporozoite proteins that are modular adhesion glycoproteins to which cellular receptors may attach.<sup>61,64</sup> The other potential adhesion sequence has partial homology to a 10-amino acid region (ie, RNIAEIKDI) contained in the  $\beta_1$ -chain of the mouse basement membrane protein, laminin.<sup>67</sup>

The extracellular region of CD34 has several potential sites for glycosylation.<sup>68</sup> There are six to nine acceptor sites

(NXS/T) for N-linked glycans, and this region is also rich in serine and threonine residues (ie, about 35% of the core protein<sup>6,41-43,69</sup>), consistent with the known extensive O-linked glycosylation of the CD34 protein. How glycosylation contributes to the function of the CD34 molecule is not clear. O-glycosylation renders some proteins relatively resistant to proteases,<sup>44</sup> and the CD34 protein is highly stable.<sup>16,57,58</sup> Furthermore, glycan chains with over 20 sugar residues are often present as constituents of mucin adhesion proteins.<sup>46</sup> CD34 is a sialomucin with heavily sialylated glycan chains.<sup>12</sup> The extended bulky conformations of these glycan-rich regions are postulated to provide an extended scaffold on which specific recognition interactions may occur.

A cysteine-rich region in the extracellular domain encoded by exons 4 and 5 is homologous to that of members of the Ig superfamily. In this region, which contains six approximately evenly spaced cysteine residues,<sup>6</sup> folding and compaction of CD34 may occur to yield a more globular structure, as is the case with Igs (Fig. 1).

Proceeding toward the carboxyl-terminal region of CD34, exon 7 encodes an hydrophobic stretch of 23 amino acids consistent with a single transmembrane domain characteristic of the type I transmembrane proteins (Fig. 1).<sup>4,41-44</sup> Finally, the cytoplasmic region of CD34, encoded by exons 7 and 8, contains the highest degree of sequence similarity between human, murine, and canine CD34. Within this region, there is greater than 90% amino acid identity, and approximately 92% nucleotide identity.<sup>4,41-49</sup> Such a high degree of conservation strongly suggests an important functional role. This region contains several known or potential protein kinase phosphorylation target sites, including two for PKC (R/K-X<sub>1,2</sub>-S/T-X<sub>3</sub>R/K) and one for GSK-3 (S-X<sub>4</sub>S) (Fig. 1). Human CD34 can be phosphorylated to high stoichiometry by activated PKC, further suggesting functional relevance for these PK sites.<sup>16</sup> Similarly, treatment of CD34<sup>+</sup> murine cell lines with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) rapidly induces phosphorylation of murine CD34.<sup>11</sup> In response to activation of PKC, normal human marrow CD34<sup>+</sup> cells and neonatal cord blood-derived human KMT2 cells rapidly upregulate surface CD34.<sup>16</sup>

## REGULATION OF CD34 GENE EXPRESSION

### Transcriptional Regulation

Regulation of the expression of CD34 occurs at both the transcriptional and posttranscriptional levels, although the mechanisms involved are not yet known. Nuclear run-on studies provide evidence that CD34 is regulated at the level of transcription.<sup>41</sup> In these studies, CD34<sup>+</sup> cell lines initiate transcription, whereas several CD34<sup>-</sup> cell lines tested have low levels or absent transcriptional initiation of the CD34 gene. The transcription start site has recently been identified in both the human and murine genes.<sup>4,41-43,67</sup> In the human gene, the 5' UTR is 258 bp long,<sup>41</sup> while in the murine gene it is 84 to 120 bp long<sup>67</sup> (D.S.K., W.S.M., unpublished findings, February 1994). The CD34 promoter region of both species lacks a classic TATA or CAAT motif. The genomic sequence upstream of the transcription start site contains consensus binding sites for several potential cis-acting DNA elements, including *myb*, *myc*, *ets*, and *myf-1*.<sup>4,41-43,67</sup>

*c-Myb* is expressed predominantly in hematopoietic cells,<sup>66</sup> while *c-myc* is expressed in most proliferating cell types.<sup>70</sup> Both gene products are known to play an essential role in normal hematopoietic growth and development.<sup>71,72</sup> The *c-myc* gene product transactivates the human CD34 promoter.<sup>73</sup> Potential physiologic activation of CD34 by *c-myc* has been shown to occur in a CD34<sup>+</sup> glioblastoma cell line, in which endogenous CD34 expression could be induced by forced expression of exogenous *myb*.<sup>73</sup> Consistent with this, murine CD34 mRNA and protein levels are also increased in a murine M1 leukemia cell line overexpressing *myb* (Krause DS, Hoffman B, May WS, unpublished findings, April 1995). However, in some cells (eg, HL60 cells), *myb* is expressed at high levels, but CD34 cannot be detected at either the mRNA or protein level.<sup>74</sup> Furthermore, *myb* null murine embryonic stem cells, in which *myb* expression has been "knocked-out" via gene targeting,<sup>75</sup> show normal CD34 transcription (Krause DS, Mucenski M, May WS, unpublished results, April 1995). Therefore, transcriptional regulatory factors other than *c-myc* are clearly necessary, and *c-myc* is not required for CD34 expression.

Recent evidence shows that the *ets-2* transcription factor can transactivate human CD34 transcription independently from *myb*.<sup>76</sup> Preliminary data indicate that MZF-1, a zinc finger protein that is upregulated during myeloid differentiation,<sup>68</sup> can bind to and regulate transcription from the human CD34 promoter.<sup>76</sup> Within hematopoietic cell lines, MZF-1 activates CD34 expression and may function to maintain CD34 expression in myeloid progenitor cells (J. Morris and R. Hromas, personal communication, June 1995). The physiologic role and mechanism(s) for regulation of CD34 transcription clearly need further elucidation. Additional transcription factors are likely to be involved and may include those having putative DNA binding elements located in the upstream region of the CD34 gene, such as *myc*, SP-1,<sup>41</sup> C-EBP, and Pu.1.<sup>77</sup>

Transient transfection analyses using reporter constructs made with different regions of the upstream genomic DNA of the human CD34 gene have identified cis-acting elements required for transcriptional regulation.<sup>41,77</sup> These upstream genomic sequences can drive CD34 gene transcription in both CD34<sup>+</sup> and CD34<sup>-</sup> cell lines. The mechanism that accounts for the absence of CD34 transcription in negative cell lines appears to require DNA sequences from the 3' end of the gene (see below). Regulation of CD34 transcription may occur as a result of changes in chromatin structure.<sup>78</sup> DNase hypersensitive sites are thought to represent structural changes in the chromatin and are often specific to cells in which the adjacent gene is expressed. DNase hypersensitive regions within 9 kb of the human CD34 gene have been identified. In addition to the DNase hypersensitive regions within the promoter region, additional sites are located approximately 3 kb upstream,<sup>78</sup> within intron 1,<sup>79</sup> and at the extreme 3' end of the gene.<sup>77</sup> The 3' region may be critical for CD34 transcription regulation, as it appears to contain a cell type specific enhancer that increases reporter gene activity in CD34<sup>+</sup> but not CD34<sup>-</sup> cell lines.<sup>77</sup> Differences in DNase hypersensitivity in CD34<sup>+</sup> compared with CD34<sup>-</sup> cells have not yet been shown. An enhancer in the murine CD34 gene has also been identified by DNase I

hypersensitivity.<sup>79</sup> This region, located 3 kb upstream of the translational start site, acts as an enhancer apparently only when incorporated into the chromatin of CD34<sup>+</sup> hematopoietic cell lines.

A potential mechanism by which CD34 transcription may be repressed in CD34<sup>-</sup> cell lines is by DNA methylation at cytosine residues located 5' of guanosine residues (CpG). A CpG-rich region or island in the human CD34 genomic DNA at the exon 1-intron 1 border has been found to be hypermethylated in two CD34<sup>-</sup> cell lines and unmethylated in two CD34<sup>+</sup> cell lines.<sup>77</sup> No further details are yet available concerning this intriguing potential mechanism for transcriptional regulation.

#### Posttranscriptional Regulation

CD34 expression may also be regulated posttranscriptionally. The initial data suggesting that human CD34 is regulated at the posttranscriptional level were obtained using the KG1a human leukemia cell line.<sup>80</sup> Induction of monocytic differentiation of KG1a cells using TPA resulted in destabilization of CD34 mRNA. The half-life of the CD34 transcript decreased from 4.5 to 2.25 hours. Destabilization of the CD34 mRNA could be due to the presence of multiple copies of an ATTTA (murine) or an ATTTTAA (human) motif in the 3' UTR.<sup>41,81-84</sup> Such AT-rich sequences are commonly associated with mRNA instability.<sup>82</sup> Although the steady-state level of transcript was decreased in these KG1a cells, there was no apparent change in the surface expression of CD34 over the 48-hour time period in which this study was performed, consistent with the fact that the CD34 protein has a long half-life.<sup>16,37,58</sup> Therefore, in studies of CD34 expression, a decrease in the steady-state level of CD34 mRNA may not immediately lead to or correlate with a decrease in CD34 protein.

An additional posttranscriptional mechanism that may result in the inhibition of CD34 expression results from aborted transcription. Using nuclear run-on assays, aborted transcription has been shown to occur in some CD34<sup>-</sup> cell lines.<sup>74</sup> In both the murine and human CD34 genes, the 5' untranslated region (UTR) of exon 1 immediately upstream from the translational start site is a GC-rich region that is predicted to have extensive secondary structure, including that for a stem loop.<sup>44,85</sup> The stem-loop structures predicted to occur in both of these species may play a role in the regulation of either transcription or translation.<sup>43</sup> Since a stem-loop hairpin structure located in the 5' UTR of certain eukaryotic genes has been shown to be able to repress translation of some mRNA species,<sup>86</sup> such secondary structure in the CD34 gene may have an analogous effect on CD34 expression. Such a mechanism could help explain the persistence of the CD34 transcript in the absence of protein in HuVEC cells after culture in vitro. These cells rapidly lose surface expression of CD34 even though CD34 mRNA can still be detected.<sup>4</sup> Thus, multiple mechanisms must be responsible for a complex process resulting in the physiologic regulation of CD34 expression.

#### Regulation of CD34 Localization

There are intracellular stores of CD34 protein that can be translocated to the plasma membrane in response to extracel-

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lular signals.<sup>14</sup> Activation of PKC induces rapid upregulation of CD34 surface expression in normal human BM cells and in the neonatal cord-blood-derived cell line, KMT2. This process occurs within minutes, and is independent of CD34 transcription or new protein synthesis, indicating that upregulation involves the translocation and recruitment of preformed intracellular stores of CD34 to the cell surface. Activation of PKC, by TPA or bryostatin, induces stoichiometric phosphorylation of CD34 at a specific PKC consensus site contained in the cytoplasmic sequence of the full-length CD34 (i.e., human ser 374<sup>14,67</sup>). A specific inhibitor of PKC, NPC,<sup>68</sup> was able to abrogate both hyperphosphorylation and upregulation of surface CD34, further indicating a functional consequence of CD34 phosphorylation.

Curiously, some CD34<sup>+</sup> cell lines apparently do not display more surface CD34 after TPA treatment, although CD34 is still hyperphosphorylated. In these cells, perhaps no intracellular stores of CD34 exist, and virtually all of the cellular CD34 is already expressed on the surface. Alternatively, an accessory phosphoprotein may be required for upregulation of CD34. A role for CD34 phosphorylation in a transmembrane signalling pathway, independent of an upregulation mechanism, may exist. PDGF and/or CSF-1 (M-CSF), in HL60 and NIH-3T3 cells, stably transfected with exogenous human CD34, induce rapid phosphorylation of this human CD34.<sup>17</sup> However, to date a physiologic mediator of CD34 upregulation (eg, a growth factor or cytokine) in CD34<sup>+</sup> progenitor/stem cells has not been identified.

Surface expression of CD34 on primary human vascular endothelial cells (HuVEC) is completely downregulated after freshly isolated cells undergo several population doublings in vitro.<sup>15</sup> However, when these same cells are inhibited from proliferating, by high-density cell contact, they fail to downregulate CD34. These data link CD34 expression to proliferation and/or cell-cell interactions. Furthermore, treatment of the HuVEC cells for 3 days with the inflammatory mediators IL-1 $\beta$ , interferon- $\gamma$  (IFN- $\gamma$ ), or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induces upregulation of the adhesion molecule ICAM-1 and downregulation of CD34 mRNA. Reciprocal regulation of these molecules indirectly implicates CD34 in a cell adhesion process, at least in endothelial cells.

Continued efforts to elucidate the regulatory mechanisms and identify the factor(s) involved in control of CD34 expression should provide important insights into how genes expressed in developmentally early stem/progenitor cells are regulated during the processes of self-renewal and differentiation. Such knowledge should facilitate attempts to optimize strategies for BMT and gene therapy with stem/progenitor cells. Such findings are also anticipated to help fill in the gaps in our knowledge concerning the fundamental mechanism(s) involved in the dysregulation of genes expressed in hematopoietic malignancies.

## POTENTIAL FUNCTIONS OF CD34

Despite its use for selection of hematopoietic progenitor and stem cells, the function of CD34 has remained elusive. Studies identifying L-selectin as a ligand for CD34, and CD34 overexpression experiments in hematopoietic cells in-

dicate a role for CD34 in cell adhesion and inhibition of hematopoiesis.

## Cell-Cell Adhesion

Specific cellular adhesion and migration of hematopoietic stem/progenitor cells is likely to be required for both embryonic hematopoietic development as well as the dynamic repopulation of hematopoiesis that occurs daily in the adult BM compartment. Thus, several laboratories are pursuing the interesting hypothesis that CD34 is involved in adhesion of hematopoietic cells to BM stroma. A role for CD34 in adhesion has been theorized because, like other known adhesion molecules, CD34 is a heavily glycosylated surface sialomucin,<sup>12,13</sup> and CD34<sup>+</sup> hematopoietic cells given intravenously are thought to be able "home" to the BM compartment for their development after BMT.<sup>4,11</sup> L-selectin, the lymphocyte homing receptor, binds to both GLYCAM I and CD34 present on murine high endothelial venule (HEV) cells in the lymph nodes.<sup>12</sup> L-selectin was shown to bind directly to GLYCAM I as a result of a protein-protein interaction, and its binding to CD34 was found to be sialic acid specific and Ca<sup>2+</sup>-dependent.<sup>12,17</sup> However, to date the L-selectin chimeric protein has not been reported to bind to CD34 present on either non-HEV endothelial tissues or hematopoietic progenitor cells. This suggests that differential glycosylation of CD34 across cell types may affect adhesive interactions with L-selectin as well as other molecules and cells. Alternatively, the presence of different accessory molecules similar to GLYCAM I may be required.

These studies raise the interesting possibility that marrow-derived CD34<sup>+</sup> hematopoietic stem/progenitor cells may localize to the marrow compartment as a result of binding to an L-selectin-like molecule. Such a "homing" mechanism might mimic the multistep process identified for leukocyte-endothelial cell interactions.<sup>69</sup> The first step, initiating cell-cell interactions, is envisioned to require interaction between CD34 and an L-selectin-like molecule, and the second step would involve stronger binding of an integrin molecule (eg, VLA-4) on the CD34<sup>+</sup> cell to its ligand (eg, VCAM-1) on the marrow stroma. This might create a stable interaction between circulating stem/progenitor cells and the BM capillary endothelium. The stem/progenitor cells, now halted in their circulation, may then pass through the endothelial layer, adhere to the BM extracellular stromal matrix, and undergo proliferation and maturation. Supporting this theory, preliminary results have shown that murine thymocytes displaying high levels of human CD34 can selectively adhere to human but not murine marrow stroma.<sup>49</sup> This interaction is due to the expression of human CD34 because normal murine thymocytes were unable to adhere to human marrow stroma. Although this intriguing preliminary finding requires further study, the implications with respect to an adhesion function for CD34 are provocative. It is possible that the differential glycosylation of CD34 mentioned above may affect its ability to bind BM stroma, and thus influence the compartmentalization of stem/progenitor cells.

## Hematopoietic Differentiation

Preliminary evidence points to a role for CD34 in preventing the terminal differentiation of myeloid cells, and

thus in maintaining the cells at an immature hematopoietic stage.<sup>18</sup> When murine myeloid M1 leukemia cells are induced to differentiate by either IL-6 or leukemic inhibitory factor (LIF), rapid downregulation of endogenous CD34 mRNA occurs.<sup>19</sup> This is followed by differentiation from blasts to morphologically mature and functionally active macrophages. Constitutive expression of recombinant full-length but not truncated mCD34 protein in M1 leukemia cells was associated with a block in the terminal differentiation program. The cells arrested at a morphologically intermediate stage in differentiation (i.e., promonoblast) and failed to acquire the functional phenotype of mature macrophages (i.e., phagocytosis<sup>18</sup>). In contrast, cells constitutively expressing the truncated form of exogenous CD34 completed the terminal differentiation process in response to cytokine induction. These data suggest that the full-length and truncated forms of CD34, which differ only in the intracellular cytoplasmic domain region (Fig 1), have functionally distinct effects on the differentiation program of a myeloid tissue culture cell. Thus, downregulation of CD34 may be necessary for differentiation, and the inappropriate or dysregulated expression of the full-length form of CD34 in leukemic cells<sup>21</sup> could contribute to their undifferentiated phenotype.

#### Preliminary Results From CD34 Knock-out Mice

Personal communication from two laboratories that have produced CD34 knock-out mice indicates that the mice are viable, and are able to reproduce (L. Lasky, personal communication, November 1994 and February 1995; T. Mak, personal communication, August 1994). Hematopoiesis in such animals appears unaffected because PB and BM morphology and differential counts are normal. However, the mice may have subtle abnormalities in hematopoiesis. Cells derived from the yolk sac of day 10.5 CD34 knock-out embryos contain two to three times fewer CFUs than normal controls. Similarly, fetal liver cells derived from day 14 and 15 embryos have twofold to threefold fewer CFUs (L. Lasky, personal communication, February 1995). This decrease in CFUs suggests that there may be a decrease in progenitor cells at some developmental stages in the CD34 knock-out mice. When adult CD34 knock-out mice are administered sublethal doses of irradiation, they recover completely, indicating that an expandable progenitor cell pool is still present. The long-term repopulating ability of hematopoietic progenitors from these mice has not yet been determined (L. Lasky, personal communication, February 1995). No abnormality in leukocyte trafficking was detectable in the CD34 knock-out mice. It is possible that a double knock-out of both CD34 and GLYCAM 1 (both ligands for L-selectin) will affect leukocyte trafficking and homing to sites of infection.

#### CLINICAL APPLICATIONS OF CD34

Even though the function of the CD34 phosphoglycoprotein has not yet been elucidated, clinical application of CD34 MoAbs and purified CD34<sup>+</sup> hematopoietic cells is already a large and rapidly expanding field. CD34 is used as a marker for leukemia diagnosis and subclassification, as a label for quantitation of stem/progenitor cells in blood and marrow,

and as a target for immunologic purification of stem/progenitor cells for clinical transplantation.<sup>24,25</sup>

#### Use of CD34 in Leukemia Diagnosis and Subclassification

Whether dysregulated expression of CD34 plays a role in leukemogenesis is an intriguing possibility. Approximately 40% of cases of acute myeloid leukemia (AML) express CD34,<sup>1,7,20-23</sup> as do the in vitro assayed colony-forming progenitor cells in chronic myelogenous leukemia (CML).<sup>21,24</sup> In AML, CD34 expression is correlated with other biologic features. Essentially all AML cases with the t(8;21) translocation (French-American-British [FAB] M2) express CD34, HLA-DR, and low levels of CD19.<sup>25</sup> AML cases with the t(8;21) karyotype have a relatively good prognosis. In addition, CD34 is coexpressed with CD2 in most cases of AML with the inversion 16 karyotype (FAB M4Eo), another relatively good prognostic AML subtype.<sup>26</sup>

CD34 is also strongly expressed in a high percent of cases of AML with myelodysplastic features and karyotypes, and in alkylating agent-associated secondary AML cases.<sup>20,23</sup> In fact, an increase in the number of CD34<sup>+</sup> cells in the marrow or blood of a patient with myelodysplasia can be used as an objective measurement predicting blast crisis.<sup>27</sup> The subtypes of AML associated with myelodysplasia have a poor prognosis. Because CD34 is expressed both in certain good prognosis and certain poor-prognosis AML subtypes, CD34 expression appears not to be an independent prognostic indicator. CD34 is also associated with other AML cases with an immature phenotype, eg, FAB M1 and terminal deoxynucleotidyl transferase<sup>positive</sup> AML.<sup>28</sup> It is tempting to classify these leukemias as "stem cell leukemias," but the importance of this designation is unclear. For example, it may be more important to consider the observation that expression of CD34 in AML is highly correlated with expression of the multiple drug resistance (MDR) protein.<sup>29</sup> Furthermore, normal CD34<sup>+</sup> stem/progenitor cells have high levels of MDR<sup>100</sup> and aldehyde dehydrogenase,<sup>101</sup> both of which can protect cells against antineoplastic chemotherapy and may be expressed by the CD34<sup>+</sup> leukemic counterparts of the normal CD34<sup>+</sup> stem/progenitor cells.

In childhood B-lineage ALL, CD34 is expressed in most cases (~70%) and is correlated with good prognosis.<sup>102,103</sup> CD34 is expressed in the minority of cases of T-lineage ALL.<sup>29</sup> Paralleling the selective expression of CD34 on normal stem and progenitor cells, CD34 is not expressed on the predominant malignant cells in cases of chronic lymphocytic leukemia or multiple myeloma, malignancies thought to originate in more mature cells.<sup>33</sup>

#### Use of CD34 to Quantitate Stem/Progenitor Cells

Flow cytometric analysis of CD34 immunostained marrow preparations can help quality control a harvested BM, as an addition to the leukocyte count, morphologic differential count, and colony-forming assay results.<sup>104,105</sup> This can provide especially important clinical information after ex vivo marrow processing, as it may assure that sufficient numbers of stem/progenitor cells remain in the intended transplant graft. The advantage of the CD34<sup>+</sup> cell count over the mor-

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phologic differential count and the colony-forming assay as its objectivity and precision, with less than 1 day turn-around time. CD34<sup>+</sup> cell counts should become progressively more automated and less expensive in future years. However, at present the test is not yet standardized among laboratories.<sup>12</sup> In addition, we do not yet know the minimum safe number of CD34<sup>+</sup> BM cells needed for clinical engraftment, and this may vary depending on the CD34<sup>+</sup> cell subset composition in a given patient (i.e., a given patient marrow may contain a relatively high frequency of CD34<sup>+</sup>, CD19<sup>+</sup> B-lymphoid progenitors, which do not contribute to generation of neutrophils or platelets, the cells needed by the patient most urgently after BM transplant).

CD34<sup>+</sup> blood cell counts are widely used to decide when to obtain "mobilized" hematopoietic stem/progenitor cells from blood.<sup>109-113</sup> In steady state, normal adult blood contains nearly undetectable CD34<sup>+</sup> cells, but after "mobilization" with hematopoietic growth factors, with or without mobilizing chemotherapy, the percentage of CD34<sup>+</sup> cells may increase to 1% to 5%. The CD34<sup>+</sup> cell count predicts the yield of progenitor cells that can be collected by leukapheresis.<sup>108-112,114,115</sup> There is a threshold number of CD34<sup>+</sup> blood cells infused to the myeloablative patient, above which prompt engraftment of neutrophils and platelets within ~1 to 2 weeks after transplantation is essentially assured. Although difficulties with inter-laboratory standardization of CD34<sup>+</sup> cell enumeration do not yet permit an exact stipulation, there is general agreement that this threshold is between 0.5 and 5 million CD34<sup>+</sup> mobilized blood cells/kg patient weight.<sup>116</sup> This threshold will become more precise with the availability of standardized CD34 reagent kits and specific software for flow cytometric quantitation of CD34<sup>+</sup> cells, as now exist for CD4<sup>+</sup> cell counting.

Much will be learned by studying the subtypes of progenitor cells in the mobilized CD34<sup>+</sup> cells. Surprisingly, these mobilized CD34<sup>+</sup> cell populations are remarkably more homogeneous than BM CD34<sup>+</sup> cells. After either colony-stimulating factor (G-CSF) or GM-CSF, with or without mobilizing chemotherapy, the mobilized blood CD34<sup>+</sup> cells are predominantly CD34<sup>+</sup>, CD13<sup>+</sup>, CD33<sup>+</sup>, CD38<sup>+</sup> myeloid progenitors. There are only tiny, difficult to quantitate fractions of progenitors of other lineages and of the earliest CD34<sup>+</sup>, CD38<sup>+</sup> stem/progenitor cells. Extremely careful analysis will be required to correlate these minor CD34<sup>+</sup> cell subsets with clinical features such as prior chemotherapy and rapidity and durability of engraftment.

### Use of CD34 MoAbs to Purify Stem/Progenitor Cells

CD34 is currently used to purify normal human stem/progenitor cells for hematopoietic rescue after high-dose myeloablative therapy in patients.<sup>117</sup> The major advantage of CD34<sup>+</sup> cell purification is the removal by positive selection of unwanted cells. As already discussed, outside of the hematopoietic system, CD34 is expressed only on endothelial cells and certain fibroblastic cell types. Similarly, with the exception of CD34<sup>+</sup> endothelial cell cancers,<sup>21,118</sup> CD34 is generally not detectably expressed in solid tumors.<sup>25</sup> Over the past several years, there has been a progressive increase in the use of high dose chemotherapy plus hematopoietic rescue

(i.e., autologous BM or blood transplant) for advanced solid malignancies. There is also increasing evidence that BM and blood frequently contain occult cancer cells which can contribute to relapse after transplant.<sup>119</sup> Therefore, in patients undergoing autologous BM or mobilized blood reinfusions, the positive selection of CD34 stem/progenitor cells may be an effective method of leaving behind or "reverse purging" the graft of occult malignant cells which do not express CD34 (eg, breast cancer, lymphomas, myelomas, ovarian cancer, neuroblastoma and other pediatric solid tumors, CD34<sup>+</sup> leukemias) and thus reduce the relapse rate.<sup>9,109,115</sup> The question of whether immunoaffinity purified CD34<sup>+</sup> populations are capable of supporting hematopoietic reconstitution in autologous transplantation is currently answered or under investigation for the various CD34<sup>+</sup> cell selection technologies.<sup>9,112,119</sup> It appears unlikely that CD34<sup>+</sup> selection alone will fully deplete the graft of cancer cells. Although the levels of tumor cells in marrow and blood harvests are still being determined, the reported trials with current CD34<sup>+</sup> selection technologies have not yet achieved consistent preparations of greater than 90% pure CD34<sup>+</sup> cells, with consequent ~3 log predicted depletion of tumor cells.<sup>120</sup> Thus, methods will need to be developed for repeated CD34<sup>+</sup> selections of the graft, or maximally effective purging of cancer cells may require a combination of positive and negative selection methodologies.

Recent reports suggest that mobilized blood allografts reconstitute hematopoiesis with a low incidence and severity of GVHD.<sup>120</sup> CD34<sup>+</sup> cell selection is being used in recently initiated trials for reverse purging of T lymphocytes from allogeneic donor mobilized blood cell grafts. Graft rejection, reported in several but not all trials using T-lymphocyte depletion of allogeneic grafts,<sup>121,122</sup> may not be a major problem when the large numbers of CD34<sup>+</sup> cells obtained from mobilized blood are administered.<sup>123,124</sup> Future studies could address the presence of a graft-versus-leukemia (GVL) effect after CD34<sup>+</sup> transplants, and even attempt to induce GVL if needed.<sup>125</sup> Demonstration of donor-derived lymphohematopoiesis years after allogeneic transplants of highly purified CD34<sup>+</sup> cells would provide final proof in humans that pluripotent lymphohematopoietic stem cells are contained in the CD34<sup>+</sup> cell population.<sup>126</sup>

CD34<sup>+</sup> selection is also in use to concentrate stem cells for gene therapy trials.<sup>127</sup> CD34<sup>+</sup> stem cell enrichment is performed to increase the retrovirus:cell ratio in the subsequent ex vivo retroviral infection protocols. This is currently necessary because high titers of the appropriate retroviruses cannot yet be obtained, and retroviral infection of stem cells is inefficient.

With the availability of many hematopoietic growth factors, it has become reasonable to consider ex vivo culture expansion of clinical transplant grafts (eg, ref 128). Of concern, current cultures result in increases in the numbers of total cells and progenitor cells, but the frequencies of CD34<sup>+</sup> cells after 1 week of culture decrease to very low levels. Thus, expansion is mainly linked to maturation, but there is also evidence in mice that sufficient pluripotent stem cells are still present in ex vivo cultured grafts.<sup>129</sup> Recently, the first clinical trial was reported using ex vivo expanded

CD34<sup>+</sup> blood cells as the autologous transplant graft.<sup>20</sup> Prompt engraftment was observed after administration to myeloablated patients of cultures initiated with about 10% of the usually required numbers of CD34<sup>+</sup> cells (ie, 0.25 million CD34<sup>+</sup> cells/kg). However, the long-term engraftment potential of these cells cannot be determined in this autologous setting. If long-term repopulating capacity can be shown, it might become possible to culture sufficient stem/progenitor cells for an average adult transplant from 500 to 1,000 mL of "mobilized" blood, thus reducing time, risk, cost, and tumor contamination of blood stem cell harvesting.

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